

REMARKS

Claims 1-6 and 12-23 constitute the pending claims in the present application. Claims 1-13 and 19 were initially elected without traverse. Claims 14-18 and 20-22 are withdrawn from consideration as being drawn to a non-elected invention. Applicants will cancel these claims upon indication of allowable subject matter in the elected invention. Claims 7-11 have been canceled without prejudice to their prosecution in this or a later-filed application. Applicants reserve the right to pursue these claims of similar or differing scope in this or a later-filed application. Claims 12-13 and 19 have been amended. Claim 23 has been added. Support for the claim amendments and new claim 23 can be found throughout the specification. No new matter is being introduced. Applicants respectfully request reconsideration in view of the following remarks. Issues raised by the Examiner will be addressed below in the order they appear in the prior Office Action.

Applicants note that the Examiner has acknowledged Applicants' election of Group I (claims 1-13 and 19) and SEQ ID NO: 23 as a sequence species, in Paper 9 filed on October 8, 2002.

Applicants note with appreciation that the species election requirement between Species A (basic region leucine zipper structures) and Species B (basic helix loop helix structures) as set forth in Paper No. 8 mailed on July 2, 2002, is vacated on the grounds that Applicants' traversal argument is found persuasive.

Applicants also thank Examiner Mahatan and Examiner Allen for the telephonic interview on June 19, 2003 and their willingness to discuss the Office Action and clarification of the claims.

Withdrawal of claims 1-13 by the Examiner

Claims 1-13 are withdrawn by the Examiner on the grounds that the elected SEQ ID NO: 23 fails to fulfill at least the defined requirement of an avian pancreatic polypeptide of claims 1-13. Specifically, the Examiner states that claims 1-13 are drawn to an avian pancreatic polypeptide (aPP), which is clearly defined in the application as "a polypeptide in which residues fourteen through thirty-two form an alpha helix stabilized

by hydrophobic contacts with an N-terminal type II polypeptide helix formed by residues one through eight” (see page 3, lines 1-10 in the specification). These claims should not be withdrawn and it appears that they were withdrawn because they were misunderstood or misread.

Contrary to the Examiner’s assertion, claims 1-13 are not drawn to “an avian pancreatic polypeptide (aPP).” Instead, these claims explicitly recite “an avian pancreatic polypeptide modified by substitution of at least one amino acid residue.” Applicants point out that the polypeptide in claim 19 (e.g., comprising SEQ ID NO: 23) is an embodiment of the modified avian pancreatic polypeptide in claims 1-13.

Applicants have amended claim 19 to clarify that the polypeptide is an avian pancreatic polypeptide which is modified by substitution of at least one amino acid residue, and which binds to a Bcl-2 protein. Such amendment is fully supported by the original specification and Figure 4. For example, as shown in Figure 4 and in the “Brief Description of the Drawings” (page 5, lines 28-29), the seven Bcl-2 binding miniature proteins including 4100 (comprising SEQ ID NO: 23), 4101 (comprising SEQ ID NO: 24), 4099 (comprising SEQ ID NO: 25), and 4102 (comprising SEQ ID NO: 26), are isolated from a BAKLIB phage library. The specification further describes how to make these Bcl-2 binding miniature proteins (see, e.g., page 14, line 21-page 15, line 12). As one skilled in the art would understand, the term “Bcl-2 protein” refers to any of a number of human proteins, including Bcl-2 itself as well as Bcl-X_L, Bid, Bax, and Bak. In particular, the specification teaches that “[i]n this procedure, the primary sequence of a protein of interest is aligned with residues in the alpha helix of aPP (emphasis added). All possible alignments of the primary sequence of positions 74-92 of Bak with aPP are assessed in two ways. First, the number of conflicts in a primary sequence alignment between residues important for hydrophobic core formation or maintenance of aPP helix dipole, and residues in Bak important for binding Bcl-X_L was considered...” Therefore, one skilled in the art readily understands that the Bcl-2 protein binding miniature proteins as shown in Figure 4 are modified avian pancreatic polypeptides.

It is evident, based on Figure 2 in the priority application No. 60/271,368, that SEQ ID NO: 23 is only a partial sequence of the modified avian pancreatic polypeptide of claim 19. Figure 2 in the priority application No. 60/271,368 was not filed in the present application. However, the priority application No. 60/271,368 is incorporated by reference in its entirety into the present application. For the convenience of the Examiner, we enclose herewith Figure 2 of the priority application No. 60/271,368 as **Exhibit 6**. As Applicants' attorney explained in the telephonic interview, some letters in Figure 2C are not in black, but rather, in colors that do not photocopy clearly. We therefore prepared a marked-up version of Figure 2C in Exhibit 6, in order to show the complete sequences (with the letters in colors underlined). We further enclose herewith **Exhibit 7** to show Figure 4 in the present application, which is identical to Figure 3 in the priority application No. 60/271,368.

As shown in Exhibit 6, the bottom sequence (labeled as BakLIB) of Figure 2C is the library from which the Bcl-2 protein binding miniature proteins (as shown in Exhibit 7, including p4099 and p4100) are derived. The listed BakLIB sequence has 34 amino acids, and contains four unspecified residues labeled as X. This sequence serves as a backbone for generating those Bcl-2 protein binding miniature proteins (including p4099 and p4100 clones). The carboxyl terminal 15 residues (residues 20-34, FVXRLLXYIXDXINR) of the BakLIB sequence is listed on top of Figure 4 (see Exhibit 7). Also shown in Exhibit 7 are the corresponding carboxyl terminal residues of the seven Bcl-2 protein binding miniature proteins that are derived from the BakLIB sequence. For example, SEQ ID NO: 23 (FVGRLRLRYFGDEINR) is listed the second from the top that corresponds to 4100. Given the sequences in the above two figures, one skilled in the art would understand that the Bcl-2 protein binding miniature proteins should have the amino terminal 19 residues (residues 1-19, GPSQPTYPGDDAPVEDLIR) of the BakLIB sequence, in addition to the 15-residue sequences listed in Figure 4 (Exhibit 7).

In addition, Applicants submit that Examples 12-16 (pages 37-42) provide detailed descriptions of making and characterizing these Bcl-2 protein binding miniature proteins. As an example of such Bcl-2 protein binding miniature protein, the sequence 4099 is described as follows: "[t]he percentage helicity of p4099 is approximately 60%,

consistent with an aPP-like tertiary fold in which residues 14-35 adopt a helical conformation...p4099 had a cooperative thermal melt with a T_m of approximately 65°C, comparable to the T_m reported for aPP” page 42, lines 5-14). Since the native aPP has 36 amino acids (see, e.g., page 3, lines 6-7; SEQ ID NO: 6; and the second sequence from the top shown in Figure 2C in the priority application No. 60/271,368), it is impossible for 4099 and other sequences (including 4100) to have only 15 amino acid residues (e.g., SEQ ID NO: 23). Instead, the length of these sequences should approximate that of aPP.

Taken together, this shows that SEQ ID NO: 23 is only a partial sequence of the modified aPP sequence in claim 19, and does not represent the whole polypeptide. The polypeptide sequence of claim 19 (comprising SEQ ID NO: 23) does fulfill the requirement of a modified aPP as specified in original claims 1-13. Therefore, Applicants respectfully request that claims 1-13 be considered in this application. Solely to expedite prosecution, Applicants have canceled 7-11 without prejudice to focus on the elected invention.

Objection to Title

The Examiner asserts that the title of the invention is not descriptive and requires a new title that is clearly indicative of the invention to the claims are directed. Specifically, the Examiner states that “the present title is directed to DNA and protein binding miniature proteins whereas in contrast the elected claims are directed to an avian pancreatic polypeptide also include constructs and recombinant host cells, but not polypeptides” (see Office Action, page 3, 9-11). The above statement is not clear to Applicants, and clarification is respectfully requested.

Applicants have elected claims 1-13 which are directed to a modified avian pancreatic polypeptide which can be either protein-binding or DNA-binding (see original claim 9 or 11 respectively). Applicants also elected claim 19 which is directed to a polypeptide relating to SEQ ID NO: 23 as a modified avian pancreatic polypeptide and a protein-binding miniature protein. Applicants will modify the title appropriately once the Examiner makes the objection clear to Applicants.

Claim rejections under 35 U.S.C. § 101 and § 112, 1st Paragraph

Claim 19 has been rejected under 35 U.S.C. § 101 because it is the Examiner's opinion that the claimed invention "lacks patentable utility due to its not being supported by either a specific, substantial utility, and credible asserted utility or a well established utility." The Examiner further rejects claim 19 under 35 U.S.C. § 112, first paragraph, for the same reasons. Applicants respectfully traverse these rejections for the reasons that follow.

The claimed invention relates to binding of a modified aPP miniature protein (e.g., comprising SEQ ID NO: 23) to a Bcl-2 protein (see, e.g., page 14, lines 21-32; page 15, lines 1-12; and Examples 13-16 on pages 38-41). It was well known in the art at the time of filing that Bcl-2 proteins (including the Bcl-2 protein, the Bcl-X_L protein, and the Bak protein) play a central role in the regulation of programmed cell death (apoptosis). It was also well known in the art that apoptosis occurs during the course of several physiological processes, and when dysregulated, contributes to many diseases including cancer, autoimmunity, and neurodegenerative disorders. See Sattler et al. 1997 (enclosed herewith as **Exhibit 1**; also referenced in the specification, e.g., page 14, lines 16-17 and lines 22-23) and Adams 2001 (enclosed herewith as **Exhibit 2**). In addition, one skilled in the art would know that "some proteins within this [Bcl-2] family, including Bcl-2 and Bcl-X_L, inhibit programmed cell death, and others, such as Bax and Bak, can promote apoptosis. Interactions between these two groups of proteins antagonize their different functions and modulate the sensitivity of a cell to apoptosis" (see **Exhibit 1**, page 983, left column).

Further, the specification clearly teaches that the modified aPP miniature protein (e.g., comprising SEQ ID NO: 23) binds to Bcl-2 and Bcl-X_L with affinities that are 100 times higher than the affinity of a peptide which is part of Bak (see, e.g., page 41, lines 1-10). In view of the teachings of the specification and the knowledge in the art, a skilled artisan would readily recognize that the modified aPP miniature protein and its variants as recited in claim 19, can potentially be used to antagonize the anti-apoptosis activity of Bcl-2 and/or exert pro-apoptosis activity of Bak, by binding to a Bcl-2 family member (e.g., the bcl-2 protein). Accordingly, a skilled artisan would appreciate the pro-apoptosis

properties of the modified aPP miniature protein and the potential of using them in the treatment of certain apoptosis-related diseases such as cancer, autoimmunity, and neurodegenerative disorders as described above.

The utility of the claimed invention is specific, as the modified aPP miniature protein (e.g., comprising SEQ ID NO: 23) specifically binds to a Bcl-2 protein. As one of ordinary skill in the art would agree, the treatment of diseases (e.g., cancer, autoimmunity, and neurodegenerative disorders) is undoubtedly a substantial utility. Additionally, the present invention has a credible utility, as one of skill in the art would know not only that a Bak-like Bcl-2 binding peptide likely promotes apoptosis, but that it is well within the scope of the skill in the art to deliver a peptide therapeutic to a patient in need of such a therapeutic. Furthermore, Applicants point out that the diagnostic and therapeutic uses for the invention are set forth at lines 18-28 on page 22 of the specification.

It is clear that the present invention is indeed supported by a specific, substantial, and credible utility, particularly as to certain diseases, such as cancer, autoimmunity, and neurodegenerative disorders. Accordingly, Applicants have satisfied the requirements under 35 U.S.C. § 101. For the same reasons, Applicants have also satisfied the requirements under 35 U.S.C. § 112, first paragraph. Reconsideration and withdrawal of the rejections are respectfully requested.

Claim rejections under 35 U.S.C. § 112, 2nd paragraph

Claim 19 is rejected under 35 U.S.C. § 112, second paragraph, as being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention. Applicants respectfully traverse this rejection to the extent it is maintained over the claims as amended.

Specifically, the Office Action asserts that claim 19 is vague and indefinite for “failing to reflect the elected SEQ ID NO: 23.” Solely to expedite prosecution, Applicants have amended claim 19 to clarify the claimed subject matter by removing the other SEQ ID NOs except SEQ ID NO: 23. Applicants reserve the right to pursue claims

of similar or differing scope. Reconsideration and withdrawal of rejections under 35 U.S.C. § 112, second paragraph, are respectfully requested.

Claim rejections under 35 U.S.C. § 102

Claim 19 is rejected under 35 U.S.C. § 102(a) and (b) as being allegedly anticipated by two Genbank sequences, Accession numbers P38803 and P43316 respectively. Specifically, the Examiner asserts that claim 19 reciting SEQ ID NO: 23 is anticipated by Genbank accession number P38803 because this Genbank sequence is a fragment of “at least twelve amino acids, has one or more conservative amino acid substitutions, and has one or more naturally occurring amino acid sequence substitutions.” Similarly, Genbank sequence P43316 allegedly anticipates claim 19 because this sequence comprises “one or more conservative amino acid substitutions, and comprises one or more naturally occurring amino acid sequence substitutions.”

First, Applicants note that the Genbank sequence Accession number P43316 appears to be incorrectly cited by the Examiner. As shown on the cited reference (page 7, left column; enclosed herewith as **Exhibit 3**), the query sequence “CYWELEW” is not part of the claimed sequence SEQ ID NO: 23. A sequence alignment of SEQ ID NO: 23 and P43316 (enclosed herewith as **Exhibit 4**) shows that the P43316 sequence is not even related to the sequence of SEQ ID NO: 23. Therefore, Applicants respectfully request that the cited P43316 should not be used as prior art against the present invention. Applicants discussed this issue with the Examiner during the telephone interview on June 19, 2003. The Examiner indicated that the cited P43316 sequence was wrong and should be withdrawn.

Second, Applicants respectfully point out that the Examiner seems to have misread section (b) of claim 19. The rejection of claim 19 under 35 U.S.C. § 102(a) appears to be based in part on the qualification of the Genbank sequence P38803 as “a fragment of at least twelve amino acids” (Office Action, page 7, line 24). In contrast, section (b) of claim 19 clearly recites “comprising a fragment of at least twelve (12) amino acids of SEQ ID NO: 23.”

Solely to expedite prosecution of the application, Applicants have amended sections (c) and (d) of claim 19, to clarify the claimed subject matter. Sections (c) and (d) of claim 19 have been amended to recite "at least 65% identical to SEQ ID NO: 23." In order for a prior art reference to be a proper reference under 35 U.S.C. § 102, the prior art reference must teach each and every element of the present invention. Claim 19 as amended, is not anticipated by either of the two cited GenBank sequences because these Genbank sequences do not teach each and every element of amended claim 19.

Applicants further point out that the above amendments are fully supported by the original specification. For example, seven isolated miniature protein sequences which are shown in Figure 4, fall in the scope of claim 19. A sequence alignment of the seven peptide sequences (enclosed herewith as **Exhibit 5**) indicates that SEQ ID NO: 23 (the partial sequence of clone 4100) shares at least 68.8% sequence identity with the other six peptide sequences listed in Figure 4. Furthermore, the specification teaches the production of a protein-binding miniature protein phage library such as the one used to produce the miniature proteins such as clones 4100 and 4099 (see Example 12, pages 37-38). The specification also describes in detail the selection of protein-binding miniature proteins (see Example 13, pages 38-39). The specification also describes how to synthesize miniature proteins such as clones 4100 and 4099 (see Example 14, pages 39-40). The specification further describes in detail how to assay the binding properties of a miniature protein, such as clones 4100 and 4099 (see Example 15, pages 40-41). Other proteins can be assessed or synthesized using this method or others available to those of skill in the art.

For the above reasons, Applicants submit that claim 19, particularly as amended, is novel and not anticipated by the cited GenBank sequences. Therefore, reconsideration and withdrawal of rejections under 35 U.S.C. § 102 are respectfully requested.

Objection to Priority

The Examiner has not granted priority to the four provisional applications (60/199,408, 60/240,566, 60/265,099, and 60/271,368), to which the present application claims priority. It appears that the Examiner raises this rejection for two reasons. First,

the Examiner states that “a sequence compliant computer readable form to which the elected SEQ ID NO: 23 can be searched” is not present in any provisional application. Second, the Examiner argues that there is no statement in any provisional application indicating the polypeptide sequence (page and line number) to which SEQ ID NO: 23 corresponds.

First, Applicants respectfully point out that 37 C.F.R. § 1.53, which sets forth the requirements for a provisional patent application, does not require a computer-readable format of the sequence listing, nor does it require that the sequences present in the provisional application be referred to by using a SEQ ID NO. In fact, the patent rules state that the provisional application is not examined. The purpose of SEQ ID NOs and a computer readable format of the sequence listing is to enable the Examiner to search the sequences of the non-provisional application which is examined on the merits. Accordingly, the provisional applications are proper and complete without any computer readable version of the sequence listing and without any identification of sequences using SEQ ID NOs, and the four above-referenced provisional applications to which the present application claims priority were properly filed.

Further, SEQ ID NO: 23 is listed as the carboxyl terminal sequence of the polypeptide sequence (labeled as No. 4100) in Figure 3 of the provisional application No. 60/271,368 filed February 23, 2001. Figure 3 of the provisional application No. 60/271,368 corresponds to Figure 4 of the instant application. Both figures list a polypeptide sequence No. 4100 that comprises SEQ ID NO: 23 as its carboxyl terminal 15 residues. Accordingly, the present application properly claims priority to the four above-referenced provisional applications, and the sequence of SEQ ID NO: 23, *per se*, is entitled to at least the filing date (February 23, 2001) of the provisional application No. 60/271,368.

Objection to Disclosure

The disclosure is objected to because of the informalities on page 1 (lines 8-12) of the specification. Applicants have amended the specification to overcome this objection.

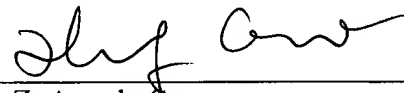
CONCLUSION

In view of the foregoing amendments and remarks, the pending claims are in condition for allowance. Early and favorable reconsideration is respectfully solicited. The Examiner may address any questions raised by this submission to the undersigned at 617-951-7000. Should an extension of time be required, Applicants hereby petition for same and request that the extension fee and any other fee required for timely consideration of this submission be charged to **Deposit Account No. 18-1945**.

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16. The protein is predicted to be largely helical [B. Rost and C. Sander, *Proteins* 19, 55 (1994)], but prediction-based threading [B. Rost, in *Protein Folds, A Distance-Based Approach*, H. Bohr and S. Brunak, Eds. (CRC Press, Boca Raton, FL, 1995), pp. 132–151; B. Rost, in *The Third International Conference on Intelligent Systems for Molecular Biology (ISMB)*, C. Rawlings et al., Eds. (AAAI Press, Menlo Park, CA, 1995), pp. 314–321] fails to identify any other proteins of similar structure; <http://www.embl-heidelberg.de/predictprotein/predictprotein.html>.
17. T. E. Creighton, *Proteins: Structures and Molecular Properties* (Freeman, New York, ed. 2, 1993).
18. A. Schöler and H. J. Schüller, *Mol. Cell. Biol.* 14, 3613 (1994); M. Profit, D. Grzesitza, K. D. Entian, *Mol. Gen. Genet.* 246, 367 (1995).
19. All yeast manipulations were in the SEY6210 background (*MATa*, *leu2-3*, *ura3-52*, *his3-Δ200*, *lys2-801*, *trp1-Δ901*, *suc2-Δ9*). The *CAT5/COQ7* locus was disrupted with a PCR-mediated approach [A. Baudin, O. Ozier-Kalogeropoulos, A. Denouel, F. La-croute, C. Cullin, *Nucleic Acids Res.* 21, 3329 (1993); the primers used were SHP84 and SHP85]. The *CAT5/COQ7* gene was entirely replaced with a DNA fragment containing a disruption module encoding the green fluorescent protein and the *HIS3* gene [R. K. Niedenthal, L. Riles, M. Johnston, J. H. Hehemann, *Yeast* 12, 773 (1996)]. Haploid cells were transformed [R. D. Gietz, R. H. Schiestl, A. R. Willems, R. A. Woods, *ibid.* 11, 355 (1995)] with the PCR product, and *HIS3* integrants were selected on minimal medium lacking histidine. Gene disruptions were confirmed by PCR analysis with primers SHP82, SHP83, and ML138. The *Δcat5/coq7* strain failed to grow (24) on YEPG or YEPE₃, which contains ethanol (71). The sequences of the primers are available on request.
20. The *CAT5/COQ7* locus was directly amplified from yeast genomic DNA by PCR with *Pfu* polymerase (Stratagene) and primers SHP69 and SHP70. A cDNA corresponding to the entire *clk-1* coding sequence was obtained by PCR amplification, also with *Pfu* polymerase, and nested primer pairs SHP57 and SHP59 and then SHP57 and SHP58 on single-stranded cDNA that had been synthesized by priming with SHP59. The respective yeast and nematode PCR products were digested with Hind III and ligated to Hind III-cut and dephosphorylated pVT102-U [T. Vernet, D. Dignard, D. Y. Thomas, *Gene* 52, 225 (1987)]. As well as restoring growth on glycerol, both the *CAT5/COQ7*- and *clk-1*-containing plasmids restored the ability of the *Δcat5/coq7* strain to grow on ethanol [YEPE₃ medium (24)]. The *Δcat5/coq7* strain transformed with the *CAT5/COQ7*-containing plasmid did not grow as well as the wild-type yeast strain on nonfermentable carbon sources. When the yeast gene was reintroduced in the context of its own promoter on a centromeric vector, full restoration of wild-type growth was obtained (24). *CAT5/COQ7* is known to be involved in the regulation of its own expression (11); presumably, the presence of excess *Cat5p/Coq7p* perturbs the normal metabolic balance of yeast. The sequences of the primers are available on request.
21. E. W. Jones, J. R. Pringle, J. R. Broach, Eds., *The Molecular and Cellular Biology of the Yeast Saccharomyces* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1992).
22. J. Campisi, *Cell* 84, 497 (1996); L. Guarente, *ibid.* 86, 9 (1996).
23. T. M. Barnes, Y. Jin, H. R. Horvitz, G. Ruvkun, S. Hekimi, *J. Neurochem.* 67, 46 (1996).
24. B. Lakowski and J. Ewbank, unpublished data.
25. By picking *Sma* non-*Dpy* recombinant progeny of *dpy-17(e164) sma-4(e729)/unc-79(e1030) clk-1(e2519) lon-1(e185)* hermaphrodites, we were able to position *clk-1* more precisely: *dpy-17 25/79 clk-1 27/79 lon-1 27/79 sma-4*. To interpolate the physical position of *clk-1*, we estimated that the separation between *ced-4* and *dpy-17* is ~0.2 centimorgans on the basis of data in the database ACeDB (7). By using linked double mutants, we also directly determined (24) the two point distances between *dpy-17*

and *sma-4* (0.85 cM), *dpy-17* and *lon-1* (0.5 cM), and *lon-1* and *sma-4* (0.35 cM). Details of the mapping data can be found in ACeDB (7).

26. The sequencing of allele *qm17*, which has a phenotype essentially identical to *e2519* (1), revealed an identical lesion. The low probability of independently obtaining the same mutation twice suggests that the original allele was lost. Sequencing of *qm47* failed to reveal a mutation. Subsequent reexamination of the phenotype of *qm47* homozygotes and new complementation tests suggest that *qm47* is not a *clk-1* allele.
27. We thank A. Coulson for cosmid; K. Kemphues for strains; J.-C. Labbé, A. Kothari, and J. Mes-Mason for nematode, mouse, and human RNA, respectively.

ly; and A. Wong, A.-M. Sdicu, and R. Durbin. Some nematode strains used in this work were provided by the Caenorhabditis Genetics Center, which is funded by the NIH National Center for Research Resources. Supported by a Royal Society–National Science and Engineering Research Council of Canada exchange fellowship and a Medical Research Council of Canada fellowship to J.J.E., a Medical Research Council of Canada grant to S.H., a Canadian Genome Analysis and Technology grant to H.B., and by fellowships to B.L. from the J. W. McConnell Foundation and Fonds pour la Formation de Chercheurs et l'Aide à la Recherche Québec.

3 September 1996; accepted 18 December 1996

GRANT-47

Structure of Bcl-x_L–Bak Peptide Complex: Recognition Between Regulators of Apoptosis

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Heterodimerization between members of the Bcl-2 family of proteins is a key event in the regulation of programmed cell death. The molecular basis for heterodimer formation was investigated by determination of the solution structure of a complex between the survival protein Bcl-x_L and the death-promoting region of the Bcl-2-related protein Bak. The structure and binding affinities of mutant Bak peptides indicate that the Bak peptide adopts an amphipathic α helix that interacts with Bcl-x_L through hydrophobic and electrostatic interactions. Mutations in full-length Bak that disrupt either type of interaction inhibit the ability of Bak to heterodimerize with Bcl-x_L.

Programmed cell death (apoptosis) occurs during the course of several physiological processes, and when dysregulated contributes to many diseases, including cancer, autoimmunity, and neurodegenerative disorders (1). The Bcl-2 family of proteins plays a central role in the regulation of apoptotic cell death induced by a wide variety of stimuli (2). Some proteins within this family, including Bcl-2 and Bcl-x_L, inhibit programmed cell death, and others, such as Bax and Bak, can promote apoptosis. Interactions between these two groups of proteins antagonize their different functions and modulate the sensitivity of a cell to apoptosis (3, 4). Several regions of the death-inhibiting proteins participate in their antiapoptotic activity and heterodimerization with the death-promoting proteins, including the Bcl-2 homology 1 (BH1) and BH2 regions (3, 5, 6). In contrast, only a relatively small portion of the

death-promoting proteins encompassing the BH3 region is critical for the ability to promote apoptosis (7–10). For example, small, truncated forms of Bak are necessary and sufficient both for promoting cell death and binding to Bcl-x_L (7).

The three-dimensional (3D) structure of the cell survival protein Bcl-x_L consists of two central hydrophobic α helices surrounded by five amphipathic helices (11). To understand how Bak interacts with Bcl-x_L and inhibits the ability of Bcl-x_L to promote cell survival, we determined the solution structure of Bcl-x_L complexed with a 16-residue peptide derived from the BH3 region of Bak. We also measured the binding affinities of Bcl-x_L to alanine mutant Bak peptides and to peptides corresponding to the BH3 regions of other Bcl-2 family members (12, 13).

The minimal region of Bak required to bind to Bcl-x_L was examined in a fluorescence-based assay (14). A 16-amino acid peptide derived from the BH3 region of Bak (residues 72 to 87) bound tightly to Bcl-x_L (Table 1). In contrast, smaller peptides from this region, such as an 11-amino acid peptide corresponding to residues 77 to 87, did not bind (Table 1). The 16-amino acid peptide of Bak corresponds precisely to the

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region of Bcl-x_L that forms the second α helix (11).

The structure of the 16-amino acid peptide (15) complexed with a biologically active deletion mutant of Bcl-x_L (16) was determined by nuclear magnetic resonance spectroscopy (NMR). The structure was determined from a total of 2813 NMR-derived restraints and is well defined by the NMR data (Fig. 1A) (17). The atomic root-mean-square deviation (rmsd) about the mean coordinate positions for residues 1 to 205 of Bcl-x_L and 72 to 87 of the Bak peptide was 0.79 ± 0.15 Å for the backbone and 1.21 ± 0.13 Å for all heavy atoms.

Overall, the structure of the truncated form of Bcl-x_L when complexed to the Bak peptide is similar to the x-ray and NMR structures of uncomplexed Bcl-x_L (11, 18). The Bak peptide binds in a hydrophobic cleft formed by the BH1, BH2, and BH3

regions of Bcl-x_L (Figs. 1 and 2). Although a random coil when free in solution (19), the Bak peptide forms an α helix when complexed to Bcl-x_L. The NH₂-terminal residues of the peptide show numerous nuclear Overhauser effects (NOEs) to residues in the BH1 region of Bcl-x_L (Val¹²⁶, Glu¹²⁹, Leu¹³⁰, and Phe¹⁴⁶), whereas the COOH-terminal portion of the Bak peptide interacts predominantly with residues in the BH2 and BH3 regions (Phe⁹⁷, Arg¹⁰⁰, Tyr¹⁰¹, and Phe¹⁰⁵). The hydrophobic side chains of the peptide (Val⁷⁴, Leu⁷⁸, Ile⁸¹, and Ile⁸⁵) point into a hydrophobic cleft of Bcl-x_L (Fig. 2) and stabilize complex formation. In addition to these hydrophobic interactions, the charged side chains of the Bak peptide (Arg⁷⁶, Asp⁸³, and Asp⁸⁴) are close to oppositely charged residues of Bcl-x_L (Glu¹²⁹, Arg¹³⁹, and Arg¹⁰⁰, respectively) (Fig. 2).

To identify the interactions that are important for complex formation, we measured the binding affinities of mutant Bak peptides containing alanine substitutions (Table 1) (14). A decrease in binding affinity by a factor of 800 was observed for the Bak peptide in which Leu⁷⁸ is substituted by an alanine. This can be explained by the loss of extensive interactions between the side chain of Leu⁷⁸ of Bak and the hydrophobic pocket formed by Tyr¹⁰¹, Leu¹⁰⁸, Val¹²⁶, and Phe¹⁴⁶ of Bcl-x_L (Fig. 2B). Mutation of other hydrophobic residues of Bak (Ile⁸⁵, Ile⁸¹, and Val⁷⁴) to alanine also resulted in reduced binding to Bcl-x_L (Table 1), which further demonstrates the importance of hydrophobic interactions in complex formation. The hydrophobic residues at these positions are largely conserved in the Bcl-2 family of proteins (Table 1). In contrast, Ile⁸⁰ is not conserved and is located on the surface of the complex (Fig. 2), consistent with the negligible loss in binding affinity observed when this residue was changed to an alanine.

Analysis of the structure (Fig. 2) suggested that the interaction between Asp⁸³ of the Bak peptide and Arg¹³⁹ of Bcl-x_L would stabilize complex formation. Indeed, Asp⁸³ is completely conserved within the Bcl-2 family of proteins, and when substituted with alanine in the Bak peptide, markedly reduced the binding of this peptide to Bcl-x_L (Table 1). Moreover, Arg¹³⁹ is highly conserved, and mutation of Arg¹³⁹ to Gln in Bcl-x_L inhibits its antiapoptotic activity and binding to the Bax protein (20). It was also expected from the structure (Fig. 2) that electrostatic interactions between Arg⁷⁶ of Bak and Glu¹²⁹ of Bcl-x_L would contribute to complex formation. This is supported by the observed decrease in binding to Bcl-x_L of a Bak peptide in which Arg⁷⁶ is mutated to alanine (Table 1).

Table 1. Binding affinities (14) of peptides to Bcl-x_L. Residues of Bak peptide substituted with alanine are in boldface. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

Peptide	Sequence	K _D (μM)
Bak	72 QQVGRQLAIIGDDINRRYDSEFQ 94	0.20 ± 0.02
	72 QQVGRQLAIIGDDINR..... 87 ←	0.34 ± 0.03
	77QLAIIGDDINR..... 87	No binding
	QQAGRQLAIIGDDINR.....	15 ± 3
	QQVGAQLAIIGDDINR.....	3.3 ± 1
	QQVGRQAALIGDDINR.....	270 ± 90
	QQVGRQLAAIGDDINR.....	1.0 ± 0.2
	QQVGRQLAIAGDDINR.....	17 ± 6
	QQVGRQLAIIGDDINR.....	0.50 ± 0.1
	QQVGRQLAIIGADINR.....	41 ± 4
	QQVGRQLAIIGDAINR.....	0.14 ± 0.02
	QQVGRQLAIIGDDANR.....	93 ± 20
Bcl-2	91 PVVHLALRQAGDDFSR..... 106	6.4 ± 0.8
Bax	57 KKLSECLKRIGDELDS..... 72	13 ± 3
Blk	55 DALALRLACIGDEMDV..... 70	15 ± 6
Bcl-x _L	84 AAVKQALREAGDEFEL..... 99	325

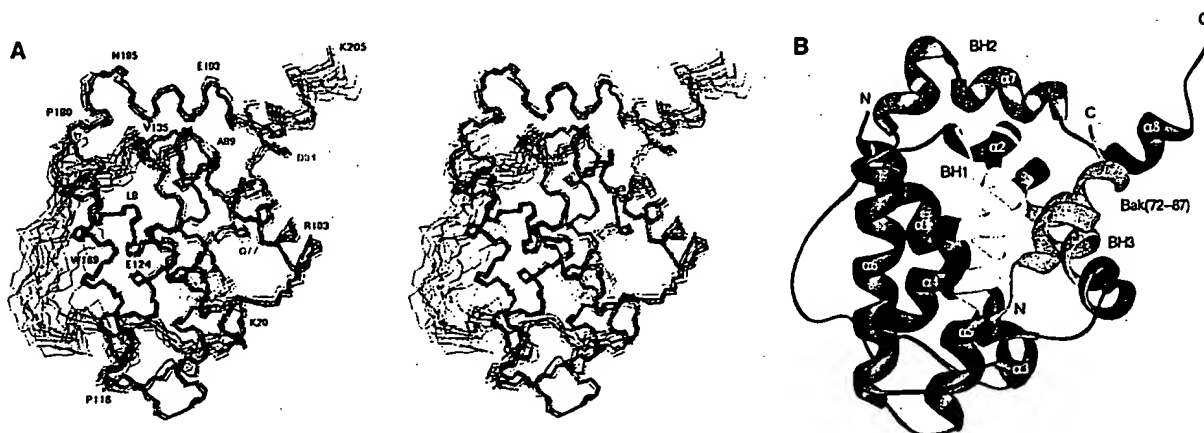


Fig. 1. (A) Stereoview of the backbone (N, C α , C') of 15 superimposed NMR-derived structures of Bcl-x_L (shown in black) complexed with the 16-amino acid Bak peptide (shown in red). (B) Ribbons (21) depiction of the

averaged minimized NMR structure for the complex. The BH1, BH2, and BH3 regions of Bcl-x_L are shown in yellow, red, and green, respectively. The Bak peptide is shown in magenta.

However, the potential charge-charge interaction between Asp⁸⁴ of Bak and Arg¹⁰⁰ of Bcl-x_L does not appear to be critical for complex stabilization as a negligible effect on binding to Bcl-x_L was observed when Asp⁸⁴ was substituted by an alanine (Table 1).

Interactions within the Bcl-2 family of proteins exhibit a defined selectivity and hierarchy (12, 13). To investigate whether this selectivity is conferred by the BH3 regions from other Bcl-2 family members, we measured the binding affinities of a series of BH3-containing peptides to Bcl-x_L (14). Subtle differences in the amino acid sequences of the BH3 regions among members of the Bcl-2 family give rise to distinct differences in the affinities of these peptides for Bcl-x_L (Table 1). The Bak peptide binds to Bcl-x_L with greater affinity than any of the other peptides, including the peptides derived from the other death-promoting proteins, Bax and Bik. The Bcl-x_L peptide binds with the weakest affinity to Bcl-x_L, consistent with the monomeric nature of this protein (11). The selectivity of Bcl-x_L that we observed for the peptides from different Bcl-2 family members is consistent with the selectivity for heterodimer formation amongst the Bcl-2 family of proteins and suggests that the BH3 region plays a central role in defining the binding specificity of the Bcl-2-related proteins for Bcl-x_L.

The molecular interactions that stabilize the Bcl-x_L-Bak peptide complex likely reflect the important interactions that occur between the full-length proteins. The wild-type Bak peptide can inhibit the interaction of Bcl-x_L with full-length Bak or Bax in a concentration-dependent manner (20). Furthermore, Bak peptides containing alanine substitutions for Leu⁷⁸ and Asp⁸³, which markedly reduced their binding to Bcl-x_L (Table 1), were unable to block heterodimer formation between full-length Bcl-x_L and Bak (Fig. 3A). When these two residues (Leu⁷⁸ and Asp⁸³) were mutated in the full-length Bak protein, the mutant Bak proteins failed to coprecipitate with Bcl-x_L even though they were expressed at levels comparable to that of the wild type protein (Fig. 3B). Thus, the reduction in binding to Bcl-x_L observed with the full-length mutant Bak proteins resembles the loss in binding to Bcl-x_L measured for the mutant Bak peptides. These data are consistent with previous reports (7–10) on the functional importance of the BH3 region of the death-promoting proteins. This region of Bak and similar sequences in Bax and Bik (Bip1) promote apoptosis and interact with Bcl-x_L (7, 8). In addition, neither the BH1 nor the BH2 region of Bax is necessary for binding to Bcl-2 or for promoting cell death (9, 10).

Using the structure of the Bcl-x_L-Bak peptide complex and a homology model of the Bak protein, we modeled the structure of the heterodimer of the full-length proteins. In the structure of Bak based on its homology to Bcl-x_L, the hydrophobic side chains of the amphipathic α 2 helix containing the BH3 region point toward the interior of the Bak protein, making these residues unavailable to interact with Bcl-x_L. Thus, binding to Bcl-x_L would necessitate a conformational change in the Bak protein to expose the hydrophobic surface of α 2. One possibility is a rotation of the α 2 helix along the helix axis that would allow the formation of the same hydrophobic and charge-charge interactions observed in the NMR structure of the Bcl-x_L-Bak peptide complex. It is of interest that based on the structure of Bcl-x_L, this helix is predicted to be flanked by highly flexible loops on both ends that could allow such a rotation.

In summary, our structure of the Bcl-x_L-Bak peptide complex reveals the structural basis for the requirements of the BH1, BH2, and BH3 regions for heterodimer formation among Bcl-2 family members. These data suggest that the formation of a hydrophobic binding cleft and properly positioned charged residues are required for the anti-apoptotic functions of Bcl-x_L. Indeed, a variety of mutations that would be predicted to alter the accessibility or binding properties of this region in Bcl-x_L and Bcl-2, including G138A (3), R139Q (20), Y101K (20), and L130A (20), have been shown to inhibit the function of this protein. For proteins that promote cell death, only the BH3 region is required for activity (7–10), which as shown here forms an amphipathic α helix and binds with high affinity to the hydrophobic groove in Bcl-x_L. Some proteins that promote cell death—such as Bik—have homology to other Bcl-2 pro-

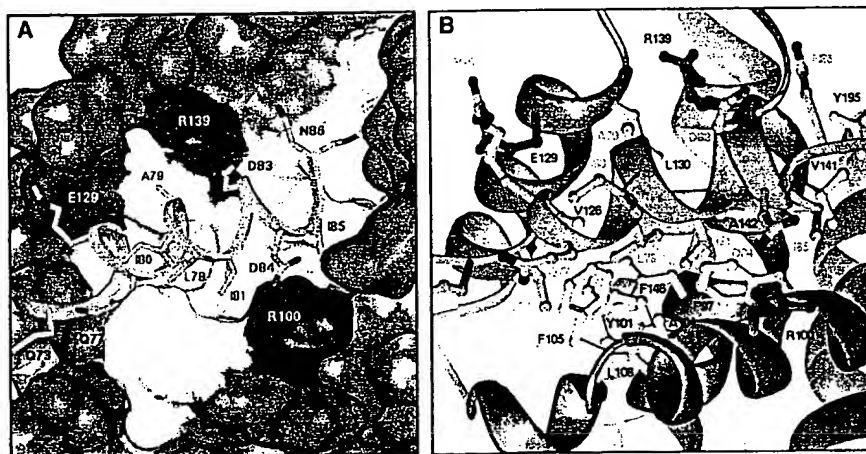
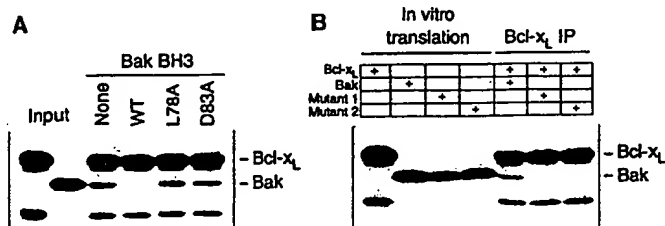


Fig. 2. (A) Surface representation of the binding pocket of Bcl-x_L bound to the Bak peptide. Hydrophobic residues showing NOEs to the peptide are colored in yellow, whereas Arg¹³⁹/Arg¹⁰⁰ and Glu¹²⁹ are colored in blue and red, respectively. Residues of Bcl-x_L are labeled in white and the Bak peptide in black. (B) Depiction of the side chains in the binding site of Bcl-x_L. Hydrophobic side chains of the protein showing NOEs to the peptide are colored in yellow. Side chains of positively and negatively charged side chains interacting with the peptide are colored in blue and red, respectively. The peptide side chains are colored by atom type. Residues of Bcl-x_L and the Bak peptide are labeled in black and green, respectively.

Fig. 3. (A) Mutations of critical residues in the Bak BH3 peptide abolish its ability to inhibit Bcl-x_L heterodimerization with Bak. In vitro-translated Bcl-x_L and Bak were combined together with 100 μ M of the indicated Bak BH3 peptide. The



reaction was immunoprecipitated with an antibody to Bcl-x (anti-Bcl-x), and the immunoprecipitated products were resolved by SDS-polyacrylamide gel electrophoresis (PAGE). (B) Mutations in Bak BH3 residues that are predicted to be involved in Bcl-x_L-Bak interactions abolish heterodimerization. In vitro-translated Bcl-x_L, Bak, or mutants of Bak were combined as indicated and immunoprecipitated with anti-Bcl-x. The immunoprecipitated products were resolved by SDS-PAGE. Bak mutation 1 contains a glutamic acid in place of arginine at amino acid 76 and an arginine in place of aspartic acid at amino acid 83. Bak mutant 2 contains an alanine in place of leucine at amino acid 78.

teins only within the BH3 region. In contrast, other Bcl-2-related proteins such as Bak or Bax are predicted to have more extensive structural similarities to Bcl-x_L. For these proteins, our studies suggest that a structural change may be required for the BH3 region to participate in dimerization.

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- The binding affinities of peptides to full-length Bcl-x_L were measured from the fluorescence emission of the Trp residues of Bcl-x_L as a function of increasing peptide concentration. The excitation and emission wavelengths were 290 and 340 nm, respectively.
- Unlabeled peptide (GVGGRQLAIIGDDINR) and a peptide uniformly ¹⁵N-, ¹³C-enriched for the Gly, Ala, Val, Leu, and Ile residues were purchased from PeptideGenic Research (Livermore, CA) and purified by reversed-phase high-performance liquid chromatography on a C8 column. NMR samples (1 to 3 mM) of a 1:1 protein-peptide complex were prepared in a 10 mM sodium phosphate buffer (pH 6.5) in ²H₂O or a 9:1 mixture of H₂O and ²H₂O.
- The deletion mutant of Bcl-x_L used in the NMR studies lacks the putative COOH-terminal transmembrane region and residues 45 to 84, which constitute a flexible loop previously shown to be dispensable for the antiapoptotic activity of Bcl-x_L (17). The deletion mutant of Bcl-x_L was constructed from the expression vector for Bcl-x_L (residues 1 to 209) (17) by a procedure similar to that of M. P. Weiner et al. [*Gene* 151, 119 (1994)]. Residue numbers correspond to full-length Bcl-x_L. Thus, in the Δ(45–84)Bcl-x_L construct used in this study, residues 44 and 85 are sequential. The Δ(45–84)Bcl-x_L construct also has four additional NH₂-terminal residues (numbers –3 to 0) due to cloning artifacts. We prepared uniformly ¹⁵N- and ¹³C-labeled proteins by growing the *Escherichia coli* strain HMS174(DE3) overexpressing Bcl-x_L on a minimal medium containing ¹⁵NH₄Cl with or without [¹³C]glucose. We prepared uniformly ¹⁵N-, ¹³C-labeled and fractionally deuterated protein by growing the cells in 75% ²H₂O. The recombinant protein was purified by affinity chromatography on a nickel-IDA column (In-vitrogen) followed by ion-exchange chromatography on an S-Sepharose column.
- NMR spectra were acquired at 30°C on a Bruker DMX500 or AMX600 NMR spectrometer. The ¹H, ¹³C, and ¹⁵N resonances of the backbone and side chains were obtained with a sample containing the (¹⁵N-, ¹³C)-labeled and 75% deuterated protein as described [T. Yamazaki, W. Lee, S. H. Arrowsmith, D. R. Muhandiram, L. E. Kay, *J. Am. Chem. Soc.* 116, 11655 (1994); G. M. Clore and A. M. Gronenborn, *Methods Enzymol.* 239, 349 (1994)]. The methyl groups of Val and Leu residues were stereospecifically assigned [D. Neri, T. Szyperski, G. Otting, H. Senn, K. Wüthrich, *Biochemistry* 28, 7510 (1989)]. Distance restraints were obtained from ¹⁵N- or ¹³C-resolved 3D NOE spectra, and φ dihedral angle restraints were measured from ³J_{H_N,H_α coupling constants [H. Kuboniwa, S. Grzesiek, F. Delaglio, A. Bax, *J. Biomol. NMR* 4, 871 (1994)]. To assign the NMR resonances of the peptide and obtain intra- and intermolecular distance restraints, we acquired 2D and 3D ¹⁵N-, ¹³C-filtered experiments on a sample with (¹⁵N-, ¹³C)-labeled protein and unlabeled peptide. Additional distance restraints from ¹⁵N- and ¹³C-separated NOE experiments were obtained with a sample of unlabeled protein complexed to the Bak peptide uniformly ¹⁵N- and ¹³C- labeled for Gly, Ala, Val, Leu, and Ile. The structure calculations were based on a distance geometry and simulated annealing protocol [J. Kuszewski, M. Nilges, A. T. Brünger, *J. Biomol. NMR* 2, 33 (1992)] with the program X-PLOR [A. T. Brünger, *X-PLOR Version 3.1*, Yale University, New Haven, CT (1992)]. NOE-derived distance restraints with a square-well potential ($F_{\text{NOE}} = 50 \text{ kcal mol}^{-1} \text{ Å}^{-2}$) were used after each was categorized as strong (1.8 to 3.0 Å), medium (1.8 to 4.0 Å), or weak (1.8 to 5.0 Å) on the basis of the NOE intensity. An additional 138 distance restraints were included for 69 hydrogen bonds identified from the slowly exchanging amides and given bounds of 1.8 to 2.3 Å (H–O) and 2.8 to 3.3 Å (N–O). No distance restraint was violated by more than 0.35 Å in any of the final structures. For the ensemble, the residual NOE rmsd was $0.009 \pm 0.003 \text{ Å}$ and the E_{NOE} was $15 \pm 3 \text{ kcal mol}^{-1}$. Torsional restraints were applied to 71 φ angles (including five for the peptide) with values of $-60 \pm 40^\circ$ ($F_{\text{tors}} = 200 \text{ kcal mol}^{-1} \text{ rad}^{-2}$) for ³J_{H_N,H_α for coupling constants <5.8 Hz in α-helical regions. No torsional angle restraint was violated by more than 5° in any of the final structures. For the ensemble, the residual torsional rmsd was $0.11 \pm 0.06^\circ$ and the E_{tors} was $0.1 \pm 0.0 \text{ kcal mol}^{-1}$. The covalent geometries were well satisfied as indicated by a small total energy ($137 \pm 10 \text{ kcal mol}^{-1}$). Although the Lennard-Jones potential was not used during any refinement stage, the final structures exhibited good van der Waals geometries as illustrated by an E_{LJ} of $-1104 \pm 12 \text{ kcal mol}^{-1}$.}}
- The rmsd between the NMR structures of free and complexed Bcl-x_L for the C^α atoms within the common regular elements of secondary structure is 1.7 Å. When complexed to the Bak peptide, residues 101 to 103 form an extension of the second α helix, the third helix in Bcl-x_L is reduced to a single helical turn, and residues 198 to 205 form an additional helix.
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- Supported in part by research grants PO1 AI35294 (C.B.T.) and R37 CA48023 (C.B.T.) from the National Institutes of Health. Coordinates for the averaged minimized NMR structure of the Bcl-x_L-Bak peptide complex have been deposited in the Brookhaven Protein Data Bank (accession number 1BXL).

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A Protein-Counting Mechanism for Telomere Length Regulation in Yeast

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In the yeast *Saccharomyces cerevisiae*, telomere elongation is negatively regulated by the telomere repeat-binding protein Rap1p, such that a narrow length distribution of telomere repeat tracts is observed. This length regulation was shown to function independently of the orientation of the telomere repeats. The number of repeats at an individual telomere was reduced when hybrid proteins containing the Rap1p carboxyl terminus were targeted there by a heterologous DNA-binding domain. The extent of this telomere tract shortening was proportional to the number of targeted molecules, consistent with a feedback mechanism of telomere length regulation that can discriminate the precise number of Rap1p molecules bound to the chromosome end.

Telomeres, the ends of linear eukaryotic chromosomes, are essential structures formed by specific protein-DNA complexes that protect chromosomal termini from degradation and fusion (1). One of the essential functions of telomeres is to allow the complete replication of chromosome ends, which cannot be accomplished by known

DNA polymerases (2). The progressive loss of DNA that would occur after each round of replication is balanced by a ribonucleoprotein terminal transferase enzyme called telomerase, which specifically extends the 3' G-rich telomeric strand in an RNA-templated reaction (3). In most organisms, telomeric DNA consists of a tandem array of short repeats. In yeast, the telomeric DNA is organized in a nonnucleosomal structure based on an array of the telomere repeat-binding protein Rap1p (4, 5).

In the human germline, cells express telomerase and maintain a constant average telomere length. This initial size appears to determine the replicative life-span of somatic cells, in which telomerase activity is usually undetectable and telomere repeats are progressively lost at each cell division (6). In unicellular organisms like *S. cerevisiae*, telomere length is kept within a narrow size distribution, specific for a given strain,

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Life-or-death decisions by the Bcl-2 protein family

Jerry M. Adams and Suzanne Cory

In response to intracellular damage and certain physiological cues, cells enter the suicide program termed apoptosis, executed by proteases called caspases. Commitment to apoptosis is typically governed by opposing factions of the Bcl-2 family of cytoplasmic proteins. Initiation of the proteolytic cascade requires assembly of certain caspase precursors on a scaffold protein, and the Bcl-2 family determines whether this complex can form. Its pro-survival members can act by sequestering the scaffold protein and/or by preventing the release of apoptogenic molecules from organelles such as mitochondria. Pro-apoptotic family members act as sentinels for cellular damage: cytotoxic signals induce their translocation to the organelles where they bind to their pro-survival relatives, promote organelle damage and trigger apoptosis.

Apoptosis, the stereotypic program of cellular suicide, removes unwanted cells throughout life, and its disrupted regulation is implicated in disorders ranging from cancer and autoimmune diseases to degenerative syndromes. The Bcl-2 family of cytoplasmic proteins plays a central regulatory role. Its interacting pro- and anti-apoptotic members integrate diverse upstream survival and distress signals to determine whether the cellular death warrant is issued. Many of the key players have been identified, and the spotlight is now on the stage where their 'dance of death' commences: the surface of organelles such as the mitochondria where Bcl-2 family members either reside or congregate during apoptosis. The hotly debated and still unresolved issue of how their control is exerted is the focus of this and other recent reviews^{1–7}.

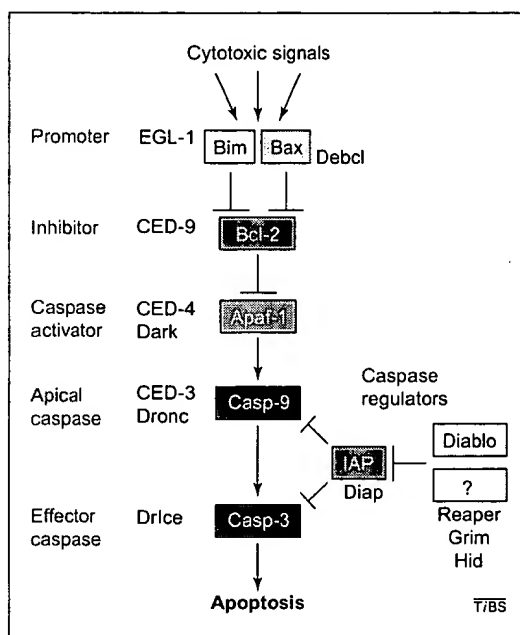
The central pathway to death

The Bcl-2 family regulates an ancient path to cell death (Fig. 1), found in organisms as diverse as mammals, nematodes and fruitflies. The route culminates in the scission of critical target proteins by proteases of the caspase group, but only after traversing critical checkpoints. To preclude unscheduled cell suicide, each caspase is synthesized as a minimally active precursor and generation of the active enzyme requires its processing, at sites of caspase cleavage. The effector caspases are processed by 'upstream' caspases, but the apical caspase that sets up the execution must process itself. The autocatalysis requires multimerization, aided by an adaptor or scaffold protein such as the nematode protein CED-4, which primes autoactivation of caspase CED-3, or the mammalian CED-4 homolog Apaf-1, which primes autoactivation of procaspase-9 (Refs 4,6,7). The Bcl-2 family determines whether or not the multimeric scaffold/procaspase complex, often termed an 'apoptosome', can assemble. Anti-apoptotic members such as Bcl-2 and its nematode counterpart CED-9 prevent apoptosome formation, but their life-saving activity can be foiled by other relatives such as Bim or EGL-1 (see below).

Caspases can also be controlled downstream of Bcl-2 (Fig. 1). The IAP (inhibitor of apoptosis) proteins appear to directly block caspase activity

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Fig. 1. The evolutionarily conserved pathway to apoptosis. Typical mammalian proteins mediating five steps to apoptosis are boxed. Their homologs are shown for *C. elegans* (all capitals) and *Drosophila*. CED-3 might serve as an effector as well as initiator caspase.



and/or activation⁸. IAP activity can in turn be blocked by other regulatory proteins. Three essential apoptogenic molecules in *Drosophila* – Reaper, Grim and Hid – can bind to IAPs and presumably unleash any associated caspase for death duty⁸. Although no mammalian Reaper-group homologs are yet known, the recently discovered mammalian Diablo (or Smac) seems to be a functional counterpart, because it can bind to IAPs and block their pro-survival activity^{9,10}.

Opposing factions of the Bcl-2 family

The diverse Bcl-2 family of proteins falls into three distinct groups. Bcl-2 and several close relatives inhibit apoptosis¹¹, whereas structurally similar relatives such as Bax³ and distant cousins such as Bik

and Bim¹² instead foster death (Fig. 2). The Bcl-2 and Bax subfamilies share three of the four conserved BH (Bcl-2 homology) sequence motifs, and can assume a similar conformation^{13–15}, but members of the third subfamily share only the BH3 domain and are otherwise unrelated even to one another. Most members of the wider clan possess a hydrophobic C-terminal segment, which facilitates their interaction with the endoplasmic reticulum (ER)/nuclear envelope and the outer mitochondrial membrane, where the pro-survival members normally reside and most others assemble during apoptosis. At least for Bax, the C terminus can also affect conformation and dimerization¹⁵.

Members of the opposing factions readily heterodimerize: the amphipathic BH3 α helix of the apoptogenic proteins can bind to a hydrophobic groove on the pro-survival proteins, created by α helices in the BH3, BH1 and BH2 regions^{13,14}. In the 'BH3-only' group, this short motif of 9–16 residues is necessary and probably even sufficient for killing activity¹². For the pro-survival proteins, however, ability to bind a BH3 domain only partly accounts for their anti-apoptotic action¹⁶, which presumably requires as-yet-unidentified effectors. Thus, a BH3-only protein apparently can serve as a ligand that locks a pro-survival protein into an inactive (or perhaps even apoptogenic) conformation. Hence, the BH3-only proteins probably kill entirely through binding other family members. Although the Bax-like proteins can also assume a conformation that permits heterodimerization via the BH3 domain, they apparently can also kill cells by an alternative mechanism, perhaps even without activating caspases (aenorhabditis)³.

Vital physiological roles

Genetic studies indicate that, without a Bcl-2-type guardian, most cells of metazoa are doomed. In *Caenorhabditis elegans*, the *ced-9* gene is required for embryogenesis, apparently because the absence of CED-9 permits CED-4 to activate CED-3 (Ref. 17). Disruption of the mammalian pro-survival genes, by contrast, primarily produces attrition only in specific tissues (reviewed in Ref. 1), presumably due to their redundancy and partially overlapping expression patterns.

The BH3-only proteins seem to be sentinels for cellular damage and critical triggers of apoptosis. In *C. elegans*, developmental cell death requires EGL-1, which can kill only through CED-9 (Ref. 18). Strikingly, mice lacking BIM exhibit elevated leukocyte numbers and succumb to autoimmune disease. Their lymphocytes display impaired responses to certain cytotoxic stimuli¹⁹. Although mice lacking BID appear normal, their hepatocytes are more resistant to apoptosis induced through the 'death receptor' CD95 (Ref. 20). Most likely, particular mammalian BH3-only proteins respond

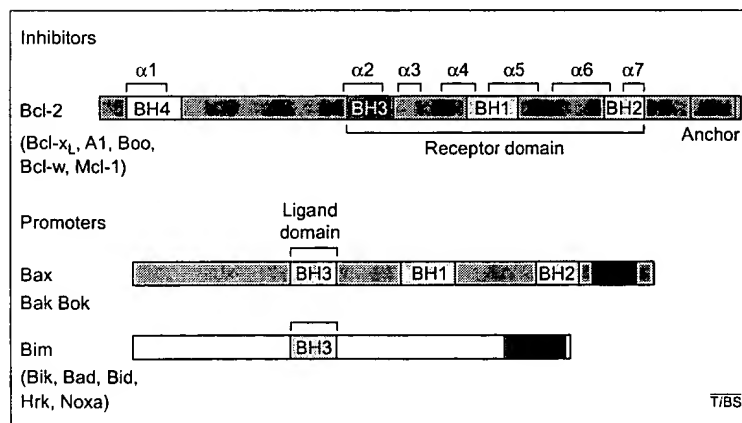


Fig. 2. Three factions of the Bcl-2 family. The conserved Bcl-2 homology (BH) domains correspond approximately to four of the seven α helices revealed by the 3D structure of Bcl-x_L (Ref. 13). The BH4 domain is confined to the pro-survival molecules most similar to Bcl-2; A1 and Boo also lack a BH3 region. A1, Bad, Bid and Noxa do not have a hydrophobic C-terminal segment (anchor). In the Bcl-2 and Bax sub-families, the pore-forming domain (see text) corresponds to the α 5– α 6 region of Bcl-x_L (Ref. 13).

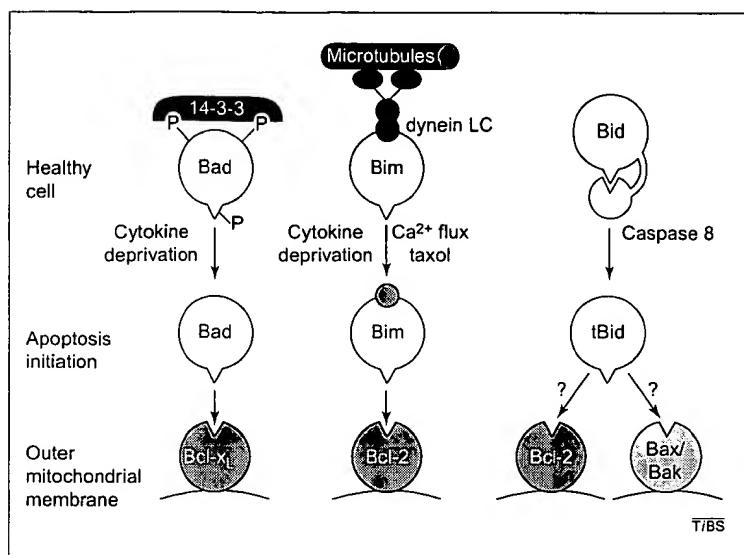


Fig. 3. Mechanisms of restraining pro-apoptotic proteins. The BH3-only proteins Bad, Bim and Bid are maintained in inactive forms in the healthy cell but unleashed by various cytotoxic signals to attack other family members and initiate apoptosis (see text).

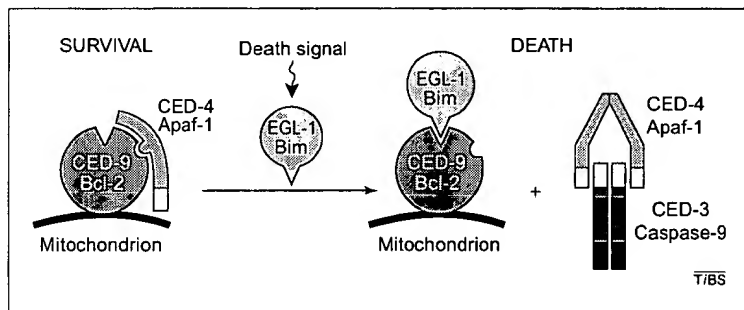


Fig. 4. Sequestration model for control of caspase activators by pro-survival family members. In *C. elegans*, all the CED-4 molecules are postulated to be held in an inactive form by CED-9 until displaced by EGL-1 and allowed to activate CED-3. However, as Bcl-2 does not appear to associate with Apaf-1, alternative models merit consideration (see text and Fig. 5).

more effectively to certain types of intracellular damage and therefore feature prominently in those responses. These potent death ligands are kept in check in different ways. Transcriptional regulation constrains the genes for EGL-1 (Ref. 18), Hrk (Ref. 21) and Noxa (Ref. 22), a target of the p53 tumor suppressor that might mediate its apoptotic function. Post-translational mechanisms control others (Fig. 3): Bid remains inert until cleaved by a caspase such as caspase-8 (Ref. 23) and Bim is restrained on microtubules by dynein light chain LC8 (Ref. 24), whereas phosphorylation of Bad on different sites allows its sequestration by 14-3-3 proteins²⁵ or directly inactivates its BH3 domain²⁶.

Because *C. elegans* lacks a Bax ortholog, the role of the Bax group is less clear. Although high levels of Bax promote cell death and some cell numbers increase in mice lacking BAX, the apoptotic responses of BAX-deficient cells are not notably diminished²⁷. Hence, it is unclear whether members of the Bax group can initiate the cell death process or instead

mainly ensure cellular demise, through mitochondrial damage, after the Bcl-2-type guardians are inactivated by BH3-only antagonists.

Translocation of Bax to mitochondria appears to require a conformational change, which might be triggered by a transient rise in pH (Ref. 28) or by Bid (Ref. 29; Fig. 3) but does not occur in cells overexpressing Bcl-2 (Refs 29,30). Altered conformation allows both Bax and Bak to homodimerize on the mitochondrial membrane and associate with Bcl-2; both these interactions are implicated in their cytotoxic action^{3,31}.

The Bcl-2 family governs the response to diverse cytotoxic conditions, including cytokine deprivation, radiation and drugs, but typically has little impact³² on the signal from 'death receptors' of the TNF family, such as CD95, which act through the adaptor FADD to activate caspase-8 (Refs 4,6,7). In some cells, however, active caspase-8 can process the BH3-only protein Bid, and the resulting Bid fragment (tBid) can trigger mitochondrial damage and activate caspase-9 (Refs 20,23). Unlike the other BH3-only proteins, tBid might activate Bax or Bak rather than inactivate a pro-survival protein (Fig. 3)^{29,31}.

Models for pro-survival function

How the pro-survival Bcl-2 family members function biochemically remains unclear. For example, it remains contentious whether they keep the caspase activators in check directly or indirectly. Results from *C. elegans* favor a direct sequestration model (Fig. 4): CED-4 normally co-localizes with CED-9 on mitochondria until a death signal induces EGL-1 to displace CED-4, which can then activate CED-3 (Refs 18,33 and references therein). It therefore seemed likely that their mammalian homologs would also associate, and Bcl-x_L was initially reported to interact with Apaf-1. Further study, however, provided no evidence for any such complexes³⁴, and indeed Apaf-1 appears to be a monomeric cytosolic protein^{4,35}. Differences in the control of CED-4 and Apaf-1 might have been expected, because only Apaf-1 contains a large C-terminal negative regulatory domain and requires cytochrome *c* as a co-factor⁴.

Nevertheless, we surmise that CED-9 and its mammalian homologs must act similarly, because a *bcl-2* transgene can rescue cells in CED-9-deficient nematodes³⁶. Perhaps the Bcl-2-like proteins sequester another mammalian CED-4 homolog, yet to be discovered, which acts upstream of Apaf-1 (Ref. 35) (Fig. 5). Additional CED-4 homologs appear likely, because the phenotype of mice lacking Apaf-1 (or caspase-9) is largely confined to neurons (reviewed in Ref. 4), and Bcl-2 can still protect Apaf-1-deficient embryonic stem (ES) cells from cytotoxic insults³⁷. Hence, it is tempting to speculate that the Bcl-2 family controls not only Apaf-1 and caspase-9 but also an unidentified scaffold protein

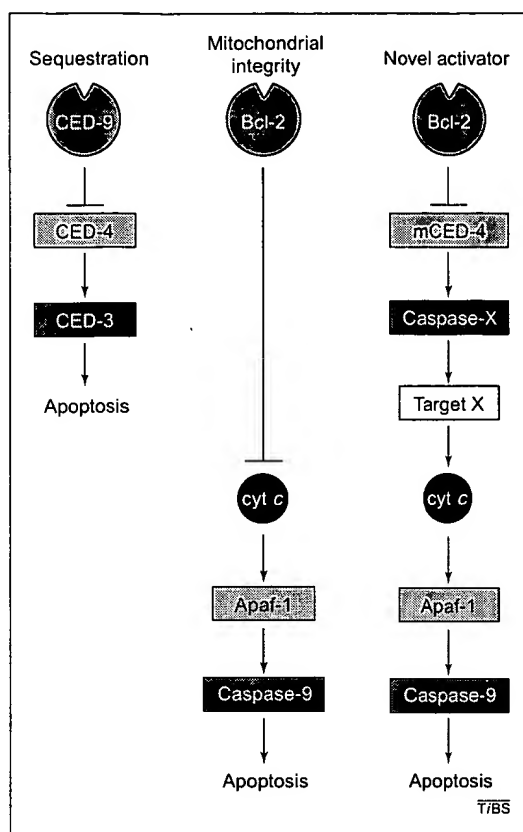


Fig. 5. Three models for pro-survival function. The sequestration model (left) favored for *C. elegans* (see Fig. 4) is compared with two models in which Bcl-2 controls Apaf-1 indirectly. In the conventional 'mitochondrial integrity' model (center), Bcl-2 directly preserves the outer membrane barrier to prevent release of cytochrome *c* (cyt *c*) and other apoptogenic molecules (see text). In the 'novel activator' model (right), Bcl-2 acts further upstream of Apaf-1 by sequestering a putative novel mammalian CED-4 homolog (mCED-4) and thereby precluding its activation of an unidentified caspase-X that can initiate apoptosis by cleaving unknown target proteins.

(mCED-4 for 'mammalian CED-4') and another upstream caspase (Fig. 5).

Impact on mitochondrial homeostasis and integrity

The alternative proposition that Bcl-2 family members govern caspase activation indirectly through effects on organelles, in particular mitochondria, is receiving increasing attention. Because Bcl-2 overexpression precludes all the mitochondrial disturbances associated with apoptosis, such as pH, membrane permeability and outer membrane integrity², Bcl-2 must either directly control the earliest of these changes (see below) and/or act at an essential even earlier step, such as release of a putative novel CED-4 homolog (mCED-4 in Fig. 5). Conceivably it does both.

The mitochondrial intermembrane space (IMS) harbors several apoptogenic proteins (Fig. 6a), and their escape probably sounds the cell's death knell. Cytochrome *c* was the first recognized IMS escapee

(reviewed in Refs 2,4), following its seminal identification as a critical co-factor (along with dATP or ATP) for the activation of caspase-9 by Apaf-1 (Ref. 38). With several cytotoxic stimuli, cytochrome *c* release precedes decreases in mitochondrial membrane potential (see below) and probably also caspase activation, because its escape is not prevented by a broad-specificity caspase inhibitor or the absence of Apaf-1 (Ref. 39). Intriguingly, a proportion of the procaspase-2, -9 and -3 molecules normally also lurk within the IMS (Ref. 40), suggesting that caspase activation could sometimes initiate inside the organelle, or concomitantly with release. Other apoptogenic escapees include the IAP inhibitor Diablo/Smac (see above) and the flavoprotein AIF (Ref. 41).

The requirement for cytochrome *c* in apoptosis was investigated recently in cell lines from mice having the gene disrupted⁴². The lines were refractory to apoptosis induced by several stimuli that affect mitochondria. Nonetheless, their substantial residual cell death is indicative of a pathway to apoptosis subject to Bcl-2 control but independent of cytochrome *c*.

How might Bcl-2-like proteins affect mitochondria? The resemblance of the 3D structure of Bcl-x_L to that in membrane-penetrating bacterial toxins prompted the hypothesis that all family members with the hydrophobic BH1-BH2 domain might form pores in organelle membranes¹³. Indeed, under certain (usually nonphysiological) conditions, Bcl-x_L, Bcl-2 and Bax can all create channels in liposomes (reviewed in Ref. 5), although there is little evidence that equivalent channels form inside cells. Conceptually, one can accept that Bax-like proteins might well damage mitochondria by pore formation, but it remains more difficult to envision how pores formed by Bcl-2-like molecules could save cells. An analogous structural domain in tBid appears to aid targeting to organellar membranes rather than to promote pore formation³¹.

Rather than forming new pores in mitochondria, Bcl-2 family members might instead stabilize or perturb the pre-existing channel through which adenine nucleotides and other small molecules traffic: the 'permeability transition pore', believed to form across sites of contact between the inner and outer membranes⁴³. The core components of this channel probably include the voltage-dependent anion channel (VDAC) in the outer membrane, adenine nucleotide translocator (ANT) in the inner one and cyclophilin D (cyclo D) in the matrix (Fig. 6a). The open channel allows passage of molecules up to 1500 Daltons, and the pore in the outer as well as the inner membrane appears to be gated^{44,45}. Opening of the inner membrane channel is thought to dissipate the H⁺ gradient across that membrane, uncoupling the respiratory chain from

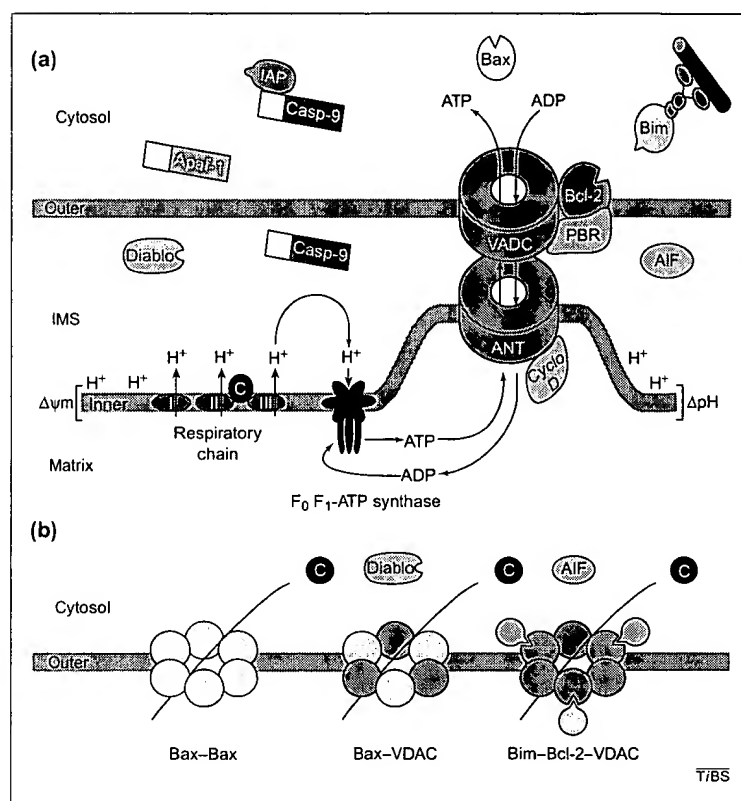


Fig. 6. How Bcl-2 family members might affect mitochondrial steps in apoptosis. (a) Putative interaction between Bcl-2 and the permeability transition pore. The pore through which adenine nucleotides presumably pass comprises VDAC and ANT; associated components might include (among others) the outer membrane peripheral benzodiazepine receptor (PBR) and cyclophilin D (cyclo D) in the matrix. Respiration creates a potential difference ($\Delta\psi_m$) across the inner membrane, and this H^+ gradient is used by the F_0F_1 -ATP synthase to drive ATP production. Bcl-2 has been reported to associate with the pore⁵⁰. Speculatively, it might keep the VDAC channel open until compromised by a molecule like Bim. (b) Hypothetical structures for the novel channel formed to allow passage of apoptogenic molecules like cytochrome c (C) and Diablo. The presumptive channel might comprise of Bax multimers, alone or associated with VDAC, or even derived from complexes that include pro-survival molecules such as Bcl-2 ligated by an apoptogenic family member.

ATP production. The resulting swelling of the matrix was proposed to rupture the outer membrane^{5,46}, but neither swelling nor rupture appear to be common^{47,48}.

Some controversial evidence suggests that certain Bcl-2 family members can associate with this mitochondrial pore and perhaps even alter its channel activity. One study suggested that Bax associated with ANT (Ref. 49), but any interaction between the normally cytosolic Bax and ANT in the inner membrane presumably would require outer membrane disruption. Another group reported that both Bcl-x_L and Bax (or Bak) could associate with VDAC and that Bcl-x_L closed a VDAC channel in liposomes, whereas Bax and Bak kept it open and allowed passage of cytochrome c (Ref. 50). More evidence is needed, however, on whether Bcl-x_L (or Bax) associates directly with VDAC and how these *in vitro* studies relate to pro-survival function *in vivo*, where Bcl-x_L might keep the outer membrane channel open rather than close it⁴⁵.

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The last rites of the cell

The order of the early steps in apoptosis remains contentious and might well vary with the death stimulus and perhaps cell type. Nevertheless, in some circumstances, the earliest observed mitochondrial changes seem to reflect closure of VDAC in the outer membrane⁴⁵. The passage of adenine nucleotides is blocked, and the potential difference across the inner membrane ($\Delta\psi_m$) transiently actually increases^{44,46}, in concert with an efflux of protons from the matrix to the cytosol⁴⁸. The cytosolic acidification, which could result from the inability of the F_0F_1 -ATP synthase to use H^+ in ATP production due to the dearth of matrix ADP, appears to precede impaired integrity of the outer membrane, caspase-9 activation and, finally, depolarization of the inner membrane⁴⁸.

In a speculative scenario, the Bcl-2 family might affect mitochondria in several ways. For example, in healthy cells, pro-survival members might associate, directly or indirectly, with VDAC and perhaps stabilize its open conformation (Fig. 6a). When a death signal has recruited BH3-only antagonists to the Bcl-2-type proteins, their interaction might switch VDAC shut. The ensuing ionic and pH changes could then prompt the translocation and oligomerization of Bax-like members. Finally, the multimerized family members, perhaps in complexes with VDAC, may create novel channels large enough to allow IMS proteins such as cytochrome c and Diablo to escape and trigger the caspase cascade (Fig. 6b). Each of these presumptive steps, however, needs much more study.

Matters of life or death

Despite the intense scrutiny of the Bcl-2 family, many critical questions remain. For the BH3-only proteins, much remains to be established about physiological roles and how damage signals are sensed. For the Bax-like proteins, issues include whether they are activated by BH3-only ligands such as Bid or by ionic changes, whether they represent additional triggers for apoptosis or an amplification mechanism, and also whether they mediate death *in vivo* primarily by compromising their pro-survival relatives, by unleashing caspases independently or by disrupting mitochondrial function. For the pro-survival proteins, key questions are how heterodimerization alters them, how their largely unexplored function on the ER and nuclear envelope relates to that on mitochondria, and, most important of all, the nature of their immediate downstream effectors: do they sequester an unidentified 'mCED-4', affect organelle pores such as VDAC or do both? Finally, an issue not discussed here, the ability of the Bcl-2 family, independent of its apoptotic role, to affect cell cycle entry remains only partly explained⁵¹. Clearly, cell death remains a lively issue!

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DR	PROSITE; PS00598		CHROMO_1; FALSE_NEG.
DR	PROSITE; PS00690		DEAH_ATP_HELICASE; 1.
DR	PROSITE; PS00013		CHROMO_2; 2.
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KW	Transcription regulation;		Repressor; Zinc-finger.
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FT	NP_BIND	755	ATP (POTENTIAL).
FT	SITE	875	DEAH BOX.
FT	DOMAIN	13	POLY-GLU.
FT	DOMAIN	16	POLY-GLU.
FT	DOMAIN	70	POLY-LYS.
FT	DOMAIN	239	POLY-GLU.
FT	DOMAIN	1279	POLY-GLU.
FT	DOMAIN	1672	POLY-ASP.
FT	MUTAGEN	737	
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53.98; Score 41; DB 1; Length 1982;

RESULT 12

Decoration 'Decoration #1': Box residues that match the Consensus exactly.

Decoration 'Decoration #1': Box residues that match the Consensus exactly.

Exhibit 5

Percent Identity

	1	2	3	4	5	6	7	
1		86.7	86.7	50.0	66.7	66.7	66.7	1
2	14.7		93.3	42.9	66.7	66.7	73.3	2
3	14.7	7.0		42.9	66.7	66.7	66.7	3
4	36.0	48.2	48.2		50.0	50.0	50.0	4
5	43.9	43.9	43.9	36.0		73.3	80.0	5
6	43.9	43.9	43.9	36.0	33.0		73.3	6
7	43.9	33.0	43.9	36.0	23.3	33.0		7
	1	2	3	4	5	6	7	

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Divergence

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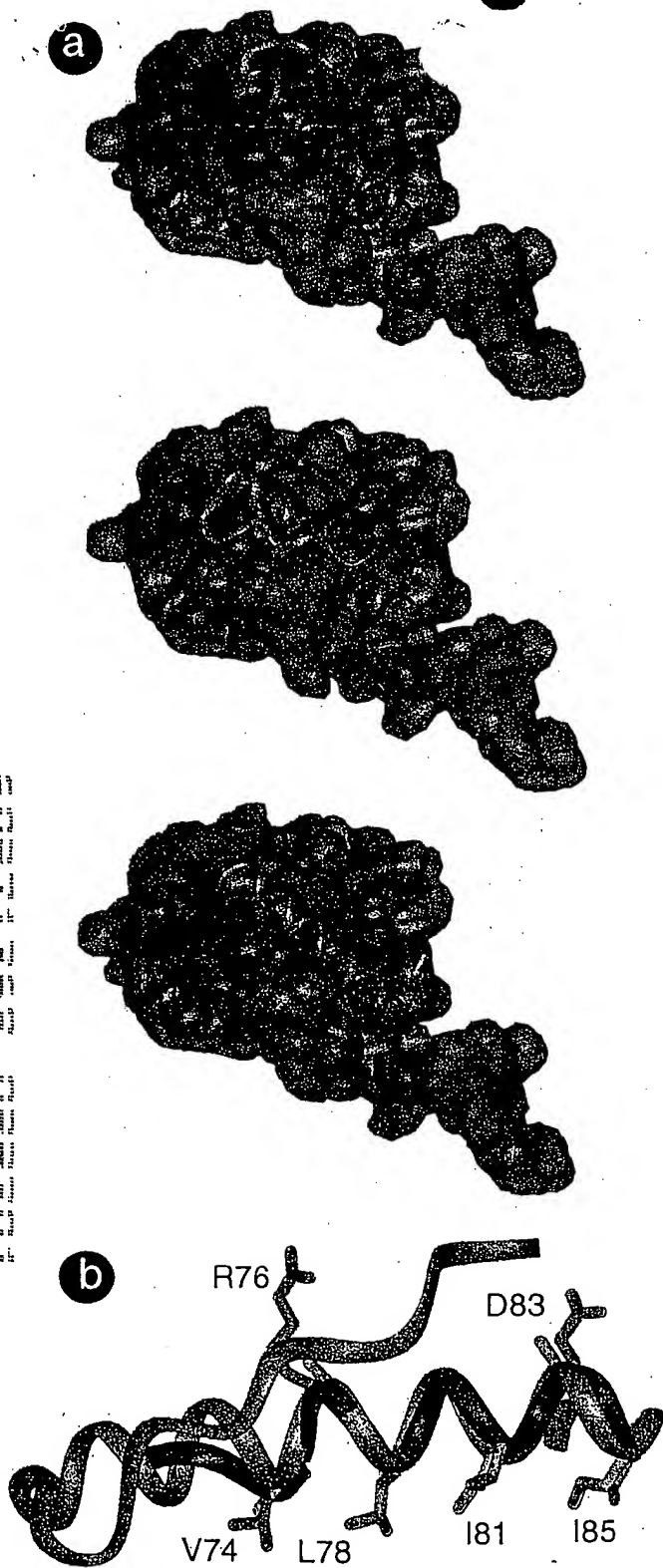
1	F	V	G	R	L	L	R	Y	F	G	D	E	I	N	R
1	F	V	G	R	L	L	A	Y	F	G	D	D	I	N	R
1	F	V	G	R	L	L	A	Y	F	G	D	T	I	N	R
1	F	V	S	R	L	L	-	R	Y	I	A	D	I	N	R
1	F	V	R	R	L	L	L	G	Y	I	D	D	I	N	R
1	F	V	L	R	L	L	L	W	Y	I	P	D	I	N	R
1	F	V	R	R	L	L	L	V	Y	I	W	D	D	I	N

Majority

SEQ ID NO_23
SEQ ID NO_24 (4101)
SEQ ID NO_25 (4099)
SEQ ID NO_26 (4102)
Figure 4_last #3
Figure 4_last #2
Figure 4_last #1

Decoration 'Decoration #1': Box residues that match the Consensus exactly.

100 101 102 103 104 105 106 107 108 109 110 111 112 113 114 115 116 117 118 119 120 121 122 123 124 125 126 127 128 129 130 131 132 133 134 135 136 137 138 139 140 141 142 143 144 145 146 147 148 149 150 151 152 153 154 155 156 157 158 159 160 161 162 163 164 165 166 167 168 169 170 171 172 173 174 175 176 177 178 179 180 181 182 183 184 185 186 187 188 189 190 191 192 193 194 195 196 197 198 199 200 201 202 203 204 205 206 207 208 209 210 211 212 213 214 215 216 217 218 219 220 221 222 223 224 225 226 227 228 229 230 231 232 233 234 235 236 237 238 239 240 241 242 243 244 245 246 247 248 249 250 251 252 253 254 255 256 257 258 259 260 261 262 263 264 265 266 267 268 269 270 271 272 273 274 275 276 277 278 279 280 281 282 283 284 285 286 287 288 289 290 291 292 293 294 295 296 297 298 299 300 301 302 303 304 305 306 307 308 309 310 311 312 313 314 315 316 317 318 319 320 321 322 323 324 325 326 327 328 329 330 331 332 333 334 335 336 337 338 339 340 341 342 343 344 345 346 347 348 349 350 351 352 353 354 355 356 357 358 359 360 361 362 363 364 365 366 367 368 369 370 371 372 373 374 375 376 377 378 379 380 381 382 383 384 385 386 387 388 389 390 391 392 393 394 395 396 397 398 399 400 401 402 403 404 405 406 407 408 409 410 411 412 413 414 415 416 417 418 419 420 421 422 423 424 425 426 427 428 429 430 431 432 433 434 435 436 437 438 439 440 441 442 443 444 445 446 447 448 449 450 451 452 453 454 455 456 457 458 459 460 461 462 463 464 465 466 467 468 469 470 471 472 473 474 475 476 477 478 479 480 481 482 483 484 485 486 487 488 489 490 491 492 493 494 495 496 497 498 499 500 501 502 503 504 505 506 507 508 509 510 511 512 513 514 515 516 517 518 519 520 521 522 523 524 525 526 527 528 529 530 531 532 533 534 535 536 537 538 539 540 541 542 543 544 545 546 547 548 549 550 551 552 553 554 555 556 557 558 559 560 561 562 563 564 565 566 567 568 569 570 571 572 573 574 575 576 577 578 579 580 581 582 583 584 585 586 587 588 589 590 591 592 593 594 595 596 597 598 599 600 601 602 603 604 605 606 607 608 609 610 611 612 613 614 615 616 617 618 619 620 621 622 623 624 625 626 627 628 629 630 631 632 633 634 635 636 637 638 639 640 641 642 643 644 645 646 647 648 649 650 651 652 653 654 655 656 657 658 659 660 661 662 663 664 665 666 667 668 669 670 671 672 673 674 675 676 677 678 679 680 681 682 683 684 685 686 687 688 689 690 691 692 693 694 695 696 697 698 699 700 701 702 703 704 705 706 707 708 709 710 711 712 713 714 715 716 717 718 719 720 721 722 723 724 725 726 727 728 729 730 731 732 733 734 735 736 737 738 739 740 741 742 743 744 745 746 747 748 749 750 751 752 753 754 755 756 757 758 759 760 761 762 763 764 765 766 767 768 769 770 771 772 773 774 775 776 777 778 779 780 781 782 783 784 785 786 787 788 789 790 791 792 793 794 795 796 797 798 799 800 801 802 803 804 805 806 807 808 809 810 811 812 813 814 815 816 817 818 819 820 821 822 823 824 825 826 827 828 829 830 831 832 833 834 835 836 837 838 839 840 841 842 843 844 845 846 847 848 849 850 851 852 853 854 855 856 857 858 859 860 861 862 863 864 865 866 867 868 869 870 871 872 873 874 875 876 877 878 879 880 881 882 883 884 885 886 887 888 889 890 891 892 893 894 895 896 897 898 899 900 901 902 903 904 905 906 907 908 909 910 911 912 913 914 915 916 917 918 919 920 921 922 923 924 925 926 927 928 929 930 931 932 933 934 935 936 937 938 939 940 941 942 943 944 945 946 947 948 949 950 951 952 953 954 955 956 957 958 959 960 961 962 963 964 965 966 967 968 969 970 971 972 973 974 975 976 977 978 979 980 981 982 983 984 985 986 987 988 989 990 991 992 993 994 995 996 997 998 999 1000



c

Bak 72-94GQVGRQLAIGDDINR

aPP GPSQPTGGDDAPVEDLIRFYNDLQQYLNVVTRHRY

align 1 GPSQPTGGDDAPVEDLIRFVGRLLAYIGDDINR

align 2 GPSQPTGGDDAPVEDLIRVGRQLAIGDVINR

align 3 GPSQPTGGDDAPVEDLIRFYVGRQLYIIVDDINR

BakLIB GPSQPTGGDDAPVEDLIRFVXRLLXYIXDXINR

marked-up sequences

Bak 72-94GQVGRQLAIGDDINR

aPP GPSQPTYPGDDAPVEDLIRFYNDLQQYLNVVTRHRY

align 1 GPSQPTYPGDDAPVEDLIRFVGRLLAYIGDDINR

align 2 GPSQPTYPGDDAPVEDLIRVGRQLAIGDVINR

align 3 GPSQPTYPGDDAPVEDLIRFYVGRQLYIIVDDINR

BakLIB GPSQPTYPGDDAPVEDLIRFVXRLLXYIXDXINR

Figure 4

BakLIB (20-36)	FVXRLLXYIXDXINR	#	Kd (nM)
4100	FVGRLLRYEGDEINR	6	401
4101	FVGRLLAYEGDDINR	2	811
4099	FVGRLLAYEGDTINR	3	352
4102	FVSRL-RYIADLINR	2	3700
	FVRLLGYIDDINR	1	
	FVLRLWYIPDGINR	1	
	FVRLLVYIWDGINR	1	